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BOVINE TUBERCULOSIS

Human multidrug-resistant *Mycobacterium bovis* infection in Mexico

Carlos A. Vazquez-Chacon [a, b], Armando Martínez-Guarneros [a, b, *], David Couvin [c], Jorge A. González-y-Merchand [b], Sandra Rivera-Gutierrez [b], Alejandro Escobar-Gutierrez [a], Juan J. De-la-Cruz López [d, e], Adriana Gomez-Bustamante [e], Gabriela A. Gonzalez-Macal [e], Livia Maria Gonçalves Rossi [a], Raquel Muñiz-Salazar [f], Nalin Rastogi [c], Gilberto Vaughan [a]

[a] Instituto de Diagnóstico y Referencia Epidemiológicos, Secretaría de Salud, Mexico City, Mexico
[b] Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City, Mexico
[c] WHO, Supranational Tuberculosis Reference Laboratory, Institut Pasteur de Guadeloupe, Abymes, France
[d] Centro Mesoamericano de Estudios en Salud Pública y Desastres, UNACH, Chiapas, Mexico
[e] Laboratorio Estatal de Salud Pública de Chiapas, SSA, Mexico
[f] Escuela de Ciencias de la Salud, Universidad Autónoma de Baja California, Ensenada, Baja California, Mexico

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SUMMARY

Here, we describe the molecular characterization of six human *Mycobacterium bovis* clinical isolates, including three multidrug resistant (MDR) strains, collected in Mexico through the National Survey on Tuberculosis Drug Resistance (ENTB-2008), a nationally representative survey conducted during 2008–2009 in nine states with a stratified cluster sampling design. The genetic background of bovine *M. bovis* strains identified in three different states of Mexico was studied in parallel to assess molecular relatedness of bovine and human strains. Additionally, resistance to first and second line anti-tuberculosis (TB) drugs and molecular identification of mutations conferring drug resistance was also performed. All strains were characterized by spoligotyping and 24-loci MIRU-VNTRs, and analyzed using the SITVIT2 (n = 112,000 strains) and SITVITBovis (n = 25,000 strains) proprietary databases of Institut Pasteur de la Guadeloupe. Furthermore, data from this study (n = 55 isolates), were also compared with genotypes recorded for *M. bovis* from USA (n = 203), Argentina (n = 726), as well as other isolates from Mexico (independent from the present study; n = 147), to determine any evidence for genetic relatedness between circulating *M. bovis* strains. The results showed that all human *M. bovis* cases were not genetically related between them or to any bovine strain. Interestingly, a high degree of genetic variability was observed among bovine strains. Several autochthonous and presumably imported strains were identified. The emergence of drug-resistant *M. bovis* is an important public health problem that jeopardizes the success of TB control programs in the region.

1. Introduction

With ~9 million new cases, including 1.6 million deaths, occurring annually, Tuberculosis (TB) is an important cause of morbidity and mortality worldwide (http://www.who.int/tb/publications/global_report/gtbr14_executive_summary.pdf?ua=1). Although TB in humans is primarily caused by *Mycobacterium tuberculosis*, *Mycobacterium bovis* is an important causal agent in developing countries [1]. Overall, *M. bovis* plays a minor role in human disease as a result of diverse preventive measures (milk pasteurization, meat inspection, etc) implemented for disease control [2]. However, some reports have suggested the re-emergence of human *M. bovis* infection, especially among high-risk populations, and a growing number of cases of invasive disease in immunocompromised patients [3].

While *M. tuberculosis* infects almost exclusively humans, *M. bovis* affects a wide range of hosts. Therefore, discriminating these two species is critical for epidemiological investigations and implementation of preventive measures aimed to control spread of

* Corresponding author. Instituto de Diagnósticos y Referencia Epidemiológicos, Francisco de P. Miranda 177 Bis, Col. Unidad Lomas de Plateros, Del. Alvaro Obregon, Mexico City 01480, Mexico. Tel.: +52 55 5062 1600x59589.
E-mail address: jarmandomartinez@yahoo.com.mx (A. Martínez-Guarneros).

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disease. Importantly, the clinical presentation caused by both mycobacteria is nearly identical, and consequently, differentiation is only possible by bacteriological and molecular methods. Advances in molecular methodologies have provided powerful tools that have helped us further our knowledge of *M. bovis* dissemination. Genetic markers such as the repeated sequences IS*6110*, DR (Direct Repeats), IS*1081*, MPTR (Major Polymorphic Tandem Repeats), PGRS (Polymorphic G1C-Rich Sequence), and MIRU (Mycobacterial Interspersed Repetitive Unit) have been used extensively to characterize mycobacterium strains and assess their genetic epidemiological relatedness as well as to identify sources of infection [4–9].

Development of multidrug-resistant TB (MDR-TB), defined as strains resistant to at least isoniazid [H] and rifampicin [R] strains, further complicates TB control [10]. MDR-TB is an increasing problem not exclusively limited to developing countries as a result of globalization, immigration from high prevalence regions and HIV co-infection [11,12]. MDR-TB outbreaks have been recognized in different regions of the world [13–16]. While most instances are associated with *M. tuberculosis*, MDR *M. bovis* outbreaks have also been reported [12,17,18]. Sporadic community cases have also been reported [11,19]. Importantly, *M. bovis* strains bear a mutation at amino acid position 169 in the *pnc*A gene conferring intrinsic pyrazinamide (Z) resistance [20].

In Latin American countries, *M. bovis* infection in humans is significantly less frequent than *M. tuberculosis*; however, underreporting and diagnostic limitations play an important role in proper identification of mycobacteria, resulting in an underestimation of the burden of *M. bovis*-related disease. Limited information is available about the transmission routes of TB in Mexico. In 2013, TB incidence and prevalence rates in Mexico were 21 and 26 per 100,000 population, respectively [21], with U.S.-Mexico border states reporting incidence rates above the national average. Bovine TB in Mexico is endemic in regions where herds are primarily dairy cattle, and as a result, the main associated risk of transmission is consumption of non-pasteurized milk and milk products [22–25]. Interestingly, no MDR *M. bovis* strains have been previously reported in Mexico.

Here, we describe the molecular characterization of six humans *M. bovis* clinical isolates collected in Mexico, including three MDR strains, through the National Survey on Drug-Resistant Tuberculosis (ENTB-2008). The genetic background of bovine *M. bovis* strains identified in three different states of Mexico is also described in this study.

2. Materials & methods

2.1. Clinical samples

M. bovis isolates were identified throughout the most recent ENTB conducted in Mexico from February 2008 to March 2009 [26]. Briefly, sample size was calculated as reported elsewhere [26]. Based on these assumptions, a sample size of 2700 cases was analyzed. Selected states included Baja California, Chihuahua, Estado de México, Guanajuato, Morelos, Querétaro, San Luis Potosí, Sinaloa and Yucatán. In each state, health jurisdictions were selected and all acid-fast bacillus (AFB) smear-positive patients were included until the target sample size was attained. A total of 2822 cases were reported, 2678 provided clinical samples and 2121 were analyzed for drug resistance. Six human *M. bovis* strains were obtained including three MDR isolates.

All strains were isolated from positive AFB samples by the corresponding local state laboratory and subsequently submitted to the National Reference Laboratory. Samples were subjected to mycobacteria isolation by culture in 12B liquid medium, Löwenstein-Jensen (LJ) and Stonebrink slants.

2.2. Mycobacterial isolation

Sputum decontamination was performed by the Petroff's sodium hydroxide method, AFB Ziehl–Neelsen staining technique followed by culture on Löwenstein–Jensen and Stonebrinck media. Classical identification using the niacin, nitrate reduction and 68 °C catalase was also performed. Cultures that remained negative after eight weeks were considered negative. Colony morphology and acid-fast bacilli identification on smears by Ziehl–Neelsen technique were performed. Bovine isolates were cultured from lymph nodes biopsies on Stonebrink medium by the Chiapas State Health laboratory.

2.3. Drug susceptibility testing

Standard testing was performed for first-line anti-TB drugs. Susceptibility profiles to first line drugs (streptomycin [S], isoniazid [H], rifampicin [R], ethambutol [E], and pyrazinamide [Z]) were determined by BACTEC 460 method (Becton Dickinson, La Jolla, CA). Susceptibility testing to second line drugs (amikacin [Amk], kanamycin [Km], capreomycin [Cm], ofloxacin [Ofx], and ciprofloxacin [Cip]), was evaluated by the Proportions Method.

2.4. Genotyping and database comparison

All strains were typed by spoligotyping [9] and 24-loci MIRU-VNTRs [27,28] as described earlier [29]. Spoligotypes in binary format were converted to an octal code for comparison with SITVIT2 proprietary database of Institut Pasteur de la Guadeloupe, which is an updated version of SITVITWEB [30], available online at http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE. At the time of this comparison, SITVIT2 contained genotyping data on almost 112,000 *M. tuberculosis* complex clinical isolates, out of which 25,000 strains were classified as *M. bovis* and further analyzed using the SITVITBovis interface available at Institut Pasteur de la Guadeloupe. In these databases, Spoligotype International Type (SIT) and MIRU International Type (MIT) designate spoligotype and MIRU patterns respectively, that are shared by two or more isolates, as opposed to "orphan patterns", which designate patterns reported for single isolates. Note that the MIT patterns were designated as 12-, 15- or 24-loci MITs depending on the MIRU-VNTR typing format used. We also used the SB number designations according to Mbovis.org (available at: http://www.mbovis.org/) to identify the *M. bovis* spoligotyping patterns in this study.

Furthermore, we also compared the test sample (Mexico this study, n = 55 isolates), with data recorded for other *M. bovis* isolates from Mexico (independent from the present study; n = 147), USA (n = 203), and Argentina (n = 726) in order to see any evidence for genetic relatedness between circulating *M. bovis* strains.

2.5. Phylogenetical analysis and geographical distribution of spoligotypes

Major phylogenetic clades lineages/sublineages in our study were determined according to the signatures provided in the SITVIT databases, and their worldwide distribution was screened both country-wise as well as macro-geographically in United Nations subregions as follows (http://unstats.un.org/unsd/methods/m49/m49regin.htm): AFRI (Africa), AMER (Americas), ASIA (Asia), EURO (Europe), and OCE (Oceania), subdivided in: E (Eastern), M (Middle), C (Central), N (Northern), S (Southern), SE (South-Eastern), and W (Western). In this classification scheme, CARIB

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